第26卷 第4期 作 物 学 报 Vol 26, No. 4
2000 年7月 ACTA AGRONOMICA SINICA July, 2000

Comparison of Culture Procedures for Regeneration of Plants from Protoplast-derived Calluses of Rice (Oryza sativa L.) X

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Abstract Four different culture procedures combined with several treating methods were compared for their effects on regeneration of plants from protoplast2derived calluses of rice. Only a small number of weak plants could be regenerated when the calluses were transferred and cultured directly from a proliferation medium containing 2, 42D to a plant regeneration medium containing BA and NAA (procedure 1). Addition of ABA to the proliferation medium induced the formation of nodular calluses with enhanced regeneration potential (procedure 2), while addition of ABA to the regeneration medium resulted in the formation of compact calluses with suppressed adventitious buds, which would grow fast upon transfer to a growth medium free of ABA (procedure 3). By Culturing of the calluses consequently on ABA supplemented proliferation medium, ABA supplemented regeneration medium and the growth medium (procedure 4), large number of more healthy plants was obtained. Statistical test indicates that procedure 2 and 3 were much more efficient than procedure 1, while procedure 4 was the most efficient for plant regeneration.

Key words Plant regeneration; Protoplast; Callus; Abscisic acid; Rice

Several years ago, our laboratory succeeded in obtaining plant regeneration of an indica rice cultivar Qiuguiai 11[1], however, the plant regeneration efficiency was very low and the majority of the regenerated plants were very weak. Through years of experiment, we have confirmed the effectiveness of several treating methods such as dehydration treatment of the calluses[2], aeration of the cultures[3, 4] and found that use of higher concentration of copper[5] was conducive to plant regeneration in rice calluses cultures.

Recently, in order to improve further the plant regeneration of the protoplast2derived calluses we have compared quantitatively and qualitatively four competent culture procedures in combination with the above mentioned treating methods. At the same time we have also investigated the effect of various concentrations of ABA in two kinds of media involved in three of the four culture procedures. Details of the experiments are presented in this paper.

1 MATERIALS AND METHODS

1.1 Protoplast-derived Calluses

Protoplasts were isolated from suspension cells originating from dehusked mature seeds of

X This research was supported by The Chinese Foundation for Agricultural Science and Education
Received on 1998211212, Accepted on 1999205214

the indica rice cultivar Qiuguiai 11 and cultured by the method described before[1]. Colonies recovered from the protoplasts were proliferated on N6 medium[6] supplemented with 2 mg·L^{-1} 2,4-D, 1000 mg·L^{-1} proline, 3% (w/v) sucrose and gelled with 0.75% (w/v) agar. Calluses grown from these colonies were used as material for evaluation of the effectiveness of the four culture procedures.

1.2 Regeneration of Plants from the Protoplast-derived Calluses

For the convenience of quantitative and qualitative comparisons among the four culture procedures, same amount of 400±10 mg fresh calluses was used as original material for every treatment in each culture procedure. To insure the reliability and reproducibility of the results all the experiments were repeated at least twice and the data were subjected to statistical analysis when possible. All cultures in the present experiments were placed under 10/14 h light/dark photoperiod of about 20 μEm^{-2}s^{-1} provided by daylight fluorescent tubes at 24~26 ℃. Names and compositions of media for culture of the calluses are given in Table 1, and four culture procedures are shown as follows:

Table 1 Supplements to media used for regeneration of plants from protoplast-derived calluses[1)]

Name of medium[2)]	Supplements (mg·L^{-1})						
	2,4-D	BA	NAA	ABA	$CuSO_4 \cdot 5H_2O$	Proline	Casein hydrolysate
PDA	2	-	-	1, 3, 6, 9	-	1000	-
PBNA	-	2	1	1, 3, 6, 9	1.25	-	500
RBN	-	2	1	-	1.25	-	500
GBN	-	0.2	1	-	-	-	500

[1)] N6 basal elements were used for all the four media. All these media contained 3% sucrose and were gelled with 0.75% agar.

[2)] PDA, a preculture medium containing 2,4-D and ABA; PBNA, a preculture medium containing BA, NAA and ABA; RBN, a regeneration medium containing BA and NAA; and GBN, a growth medium containing BA and NAA.

Procedure 1. Calluses were dehydrated (Tsukahara and Hirosawa, 1992) → calluses were cultured on RBN (30 days) → regenerated plants and shoots were transferred to GBN (20 days);

Procedure 2. Calluses were cultured on PDA (20 days) → calluses were dehydrated → calluses were cultured on RBN (30 days) → regenerated plants and shoots were transferred to GBN (20 days);

Procedure 3. Calluses were dehydrated → calluses were cultured on PBNA (30 days) → calluses with or without shoots (buds) were transferred to GBN (20 days);

Procedure 4. Calluses were cultured on PDA (20 days) → calluses were dehydrated → calluses were cultured on PBNA (30 days) → calluses with or without shoots (buds) were transferred to GBN (20 days).

1.3 Evaluation of Plant Regeneration

Number of regenerated plants was counted at the end of each experiment, and the quality of the regenerated plants was evaluated by weighing the regenerated plants and by calculating the survival rate of the regenerated plants after a 10 days' liquid culture period. 1/3 strength N6 salts solution of macroelements was used for the liquid culture.

2 RESULTS

2.1 Plant Regeneration by Procedure1

Calluses cultured by procedure 1 regenerated only a very small number of plants on RBN medium. Because most of these plants did not have sufficient roots and were small and very weak, a subculture on GBN medium was necessary. Upon transfer to GBN medium, there was only a low rate of the plants could resume sustained growth.

Table 2 Effect of various concentrations of ABA in PDA medium[1)]

Concentration of ABA in PDA (mg/L)	Callus growth on PDA (mg f. wt./flask)	No. plants regenerated on RBN
0.0	3324 a[2)]	23 c
1.0	3355 a	25 c
3.0	3008 a	46 b
6.0	2116 b	89 a
9.0	1523 c	83 a

[1)] All calluses on PAD were used for culture on RBN medium.

[2)] Data followed by the same letter are not significantly different ($p > 0.05$) according to the t-test. The same as in Table 3 and 4.

2.2 Plant Regeneration by Procedure 2

The effects of ABA at concentrations of 1, 3, 6 and 9 mg/L in the PAD medium on cultures were compared and the medium with no ABA supplement was used as the control. The result of this experiment is shown in Table 2. Concentrations of ABA at 1 and 3 mg/L had only slight effect on proliferation of the calluses and on plant regeneration in the subsequent culture. On media with higher concentrations of ABA, the calluses became more compact and nodular in appearance. Prominent effects of enhancement on plant regeneration of the calluses were observed in treatments using ABA at concentrations of 6 and 9 mg/L. Moreover, root development of the regenerated plants was also improved by the addition of ABA at the higher levels in PAD medium.

2.3 Plant Regeneration by Procedure 3

The effects of ABA at concentrations of 1, 3, 6 and 9 mg/L in the PBNA medium on cultures were compared and the medium with no ABA supplement was used as the control. The result of this experiment is shown in Table 3. In the control culture the plant regeneration was just as that by the procedure 1: only a small number of weak plants regenerated. Supplement of 1 mg/L ABA to the medium resulted in the regeneration of some stronger plants. On media with ABA at 3 and 6 mg/L, calluses regenerated suppressed buds. Upon transfer to GBN medium, these buds burst into fast growth. The concentration of 9 mg/L seemed too high: calluses on this medium produced apparently less amount of only heavily suppressed buds. The effects of BA in GBN medium were also tested. It was found that BA concentration of 0.2 mg/L was superior to 0.0 or 1.0 mg/L: without BA supplement the number of regenerated plants

Table 3 Effect of various concentrations of ABA in PBNA medium[1)]

Concentration of ABA (mg/L) in PBNA	Callus growth on PBNA (mg f. wt./flask)	No. plants regenerated on GBN
0.0	8244 a	8 c
1.0	7277 a	21 bc
3.0	5715 b	51 a
6.0	4114 c	62 a
9.0	2156 d	36 b

[1)] All calluses with shoots and buds on PBNA were used for culture on GBN.

was slightly decreased, while at 1.0 mg·L level inhibition to the growth of the roots became very evident.

2.4 Plant Regeneration by Procedure 4

The effects of various concentrations of ABA in PDA and PBNA media on plant regeneration were comparatively investigated. Calluses cultured on PDA medium of each treatment were collected and then divided evenly into three groups and cultured respectively onto each of the three kinds of PBNA media. Data for number of regenerated plants of the experiment are shown in Table 4. The best result of plant regeneration was obtained by culturing the calluses first on PDA medium with ABA at 6 mg·L and then on PBNA medium with ABA at 3 mg·L. Moreover, with ABA at 3 or 6 mg·L in PBNA medium, development of the roots of the regenerated plants was greatly enhanced in GBN medium.

Table 4 Effect of various concentrations of ABA in PDA and PBNA media[1)]

Concentration of ABA in PBNA (mg·L)	Concentration of ABA in PDA (mg·L)		
	1.0	3.0	6.0
1.0	15 Bb[2)]	19 Bb	49 Ca
3.0	63 Ac	97 Ab	121 Aa
6.0	75 Aa	80 Aa	77 Ba

1) All calluses on each PAD media were evenly divided into three groups and cultured on the three respective PBNA media.

2) The capital letters indicate the significance among rows and the small letters among columns.

2.5 Quantitative and Qualitative Comparisons of the Four Procedures

The effects of the four culture procedures were compared quantitatively and qualitatively. For quantitative comparison, data for the best treatment in each culture procedure were collected. And for qualitative comparison, the mean weight of the regenerated plants and the number of survived plants after the liquid culture period were used. Results of the comparisons shown in Table 5 clearly indicate that procedure 1 was the most inefficient, while procedure 3 is obviously more efficient than procedure 2 because procedure 3 had more survival plants as well as required shorter time for the culture. Procedure 4 was no doubt the most efficient one, by which the fresh weight per regenerated plant was doubled and the number of survival plants was increased by nearly 150 times, respectively, in comparison with the control (procedure 1).

Table 5 Comparison of the effects on plant regeneration of the four culture procedures[1)]

Procedure	No. days Required	No. plants regenerated	Mean f. wt. ·plant (mg)	No. plants survived[2)]
1	55	8	61	2 (25.3)
2	75	89	97	36 (40.5)
3	55	62	144	47 (75.8)
4	75	363	157	291 (80.2)

1) Data in this table are taken from the most proper treatment in each culture procedure. The data for procedure 1 are taken from the control in Table 3, and the data for procedure 4 have been revised from Table 4 by timing 3 to represent the amount of plants originated from 400±10 mg material calluses as in the other treatment.

2) Obtained after a 102day's liquid culture period. Data in parentheses are the percentages.

3 DISCUSSION

Regeneration of plants from protoplast2derived calluses is an indispensable step in a breeding program using protoplasts for uptaking foreign genes or by protoplast fusion. In rice, although

high efficient plant regeneration in protoplast cultures has been reported[7, 8], we believed that poor regeneration ability of the calluses and poor quality of the regenerated plants are still two frequent and main problems to many breeders. In the present experiments by comparing four competent culture procedures, we have screened a highly reproducible and efficient culture procedure for regeneration of plants from protoplast2derived calluses of rice.

Among the four culture procedures in the present studies, procedure 1 (direct transfer of calluses from a medium containing 2, 42D to a medium containing BA or kinetin) was a simple and the most common culture procedure used in regeneration of plants from rice calluses of various origin including those derived from protoplasts[9~11]. Procedure 2 was almost the same as the "two2step culture method" described by Inoue and Maeda[12], procedure 3 was similar to that adopted by Yin et al [13], and Mei et al [14], and procedure 4 was modified from a culture method designed by Yang et al [15], with main differences in using agar2gelled medium instead of their liquid medium in the first culture step and by omitting potato extract in the next step culture medium. For convenience and in reference to the two2step culture method, we named the procedure 4 a "three2step culture procedure."

Except procedure 1, the other three procedures employed ABA. The enhancing effect of ABA on plant regeneration culture of rice callus has been described and some of the possible functions in the culture have been discussed (Inoue and Maeda, 1981; Torrizo and Zapata, 1986). By the application of ABA in the culture media, Yang et al [5] and Yin et al [13] had succeeded in obtaining plants which could otherwise not be regenerated by the common culture method. Results of the present experiments not only confirmed the positive effect of ABA on enhancing plant regeneration, but also clearly demonstrated the effect of ABA on improving the quality of the regenerated plants. Thus the three2step culture procedure had satisfactory results. Nevertheless, the great contribution to the regeneration of plants by the adopting dehydration of the calluses, aeration of the cultures and supplement of larger amount of copper to the medium, also deserved to be mentioned.

Some of the plants regenerated were grown to maturity and the fertility was investigated. It was found that the fertility of the regenerated plants dropped considerably in comparison with that of plants growing from seeds, however, little difference in fertility was recognized among groups of plants regenerated by the four culture procedures. This observation indicates that the three2step culture procedure has higher application potential in practical breeding of rice.

Acknowledgements

We thank Dr. Wu Xinrong for his skillful work on culture of the protoplasts, and thank Miss Zheng Yingdong for her efficient assistance in the experiments.

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水稻原生质体愈伤组织再生植株培养程序的比较

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提 要 在4种不同的培养程序中应用了几种处理方法, 并对其在诱导水稻原生质体起源的愈伤组织再生植株中的效果进行了比较。直接将愈伤组织从含有2, 42D 的增殖培养基转移到含有BA 和NAA 的植株再生培养基上培养, 只能得到少量的弱苗(第1种程序)。在增殖培养基中添加ABA 诱导了结节状的愈伤组织形成, 使愈伤组织的植株再生能力明显加强(第2种程序); 而在植株再生培养基中添加ABA 则使愈伤组织变得紧结并形成生长受抑制的不定芽, 当这些愈伤组织被转移到不含ABA 的生长培养基后, 不定芽开始快速生长(第3种程序)。先将愈伤组织培养在含有ABA 的增殖培养基上, 然后相继转移到含有ABA 的植株再生培养基和生长培养基上, 可以取得大量健壮的再生苗(第4种程序)。统计结果显示, 第2和第3种程序的培养效果比第1种程序要好, 而第4种程序的培养效果则比其他程序好得多。

关键词 植株再生; 原生质体; 愈伤组织; 脱落酸; 水稻